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A Rapid and Simple DNA Extraction Procedure to Detect *Salmonella* spp. and *Listeria monocytogenes* from Fresh Produce Using Real-time PCR

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Abstract DNA isolation procedures significantly influence the outcome of PCR-based detection of human pathogens. Unlike clinical samples, DNA isolation from food samples, particularly from fresh and fresh-cut produce has remained a formidable task and has hampered the sensitivity and accuracy of molecular methods. We utilized a commercially available filter-based DNA isolation method (FTA) in conjunction with real-time PCR-based detection of *Salmonella* spp. and *Listeria monocytogenes*. The protocol uses filter paper discs impregnated with a patented chemical formulation that lyses cells, immobilizes DNA, and protects it from degradation. Use of the FTA method in conjunction with real-time PCR for the detection of *Salmonella* spp. and *L. monocytogenes* was compared with two commercially available DNA isolation procedures that are commonly used for high throughput real-time PCR pathogen detection systems. Both pathogens were successfully detected from artificially inoculated fresh and fresh-cut produce such as alfalfa sprouts, cilantro, green onion, broccoli, prepacked mixed salad, and spinach at low cell numbers (four to seven colony forming units per 25 g initial inoculum level before enrichment). The FTA protocol had distinct advantages of simplicity, biosafety, and compatibility for high throughput screening. This DNA preparation protocol was rapid, sensitive, required minimal handling, and reduced interference from produce-associated inhibitors of real-time PCR.

Keywords Pathogen Detection · Food-borne · Food Safety · Fresh Produce · Food Microbiology

Introduction

Advances in molecular biology have led to the use of real-time PCR as an efficient and reproducible method for detecting pathogens (McKillip and Drake 2004; Rijpens and Herman 2002; Rodriguez-Lazaro et al. 2004a, 2007). Rather than relying on culture and biochemical properties, PCR-based assays offer more rapid, sensitive, and specific detection capabilities. Conventional and well-established technologies such as single- and double-step enrichment of human pathogens combined with immunomagnetic separation have given promising results (Jaykus 2003; Fluit et al. 1993; Shelton and Karns 2001; Josefsen et al. 2007). However, these techniques tend to be labor-intensive and require a minimum of 5 to 7 days to complete the analysis in comparison to 1 to 2 days needed for conventional PCR-based assays (Josefsen et al. 2007; Wolffs et al. 2006). Real-time PCR technology can further reduce the overall detection times by replacing time-consuming post amplification electrophoresis or hybridization methods with TaqMan- or molecular beacon-based detection chemistries (McKillip and Drake 2004; Rijpens and Herman 2002;

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Rodriguez-Lazaro et al. 2007; Shelton and Karns 2001). As both detection approaches involve the use of a third oligonucleotide probe that must also anneal to the target DNA sequence, they offer improved detection specificity.

Benefits of having rapid, sensitive, and specific diagnostic tests for the detection of food-borne pathogens in food with limited shelf-life are obvious and far-reaching. However, when compared with clinical diagnosis, there are several challenges associated with microbial detection in foods, and the greatest challenge may rest in separating target DNA from inhibitory compounds in a food matrix (Jaykus 2003; Rodriguez-Lazaro and Hernandez 2006; Jofre et al. 2005; Bhagwat et al. 2008). Because of the complexity of ingredients involved in food samples, DNA preparation methods must be optimized for each food commodity (Heller et al. 2003; Grant 2003). This may especially be true for raw, ready-to-eat fresh produce which has a short shelf-life and which is rich in polyphenolics, pigments, high residual microflora, and does not under go a kill-step before consumption. Extraction methods that work for one pathogen in a particular food variety may not work for another food type (Espy et al. 2006). Although several commercial DNA extraction kits are available for clinical and environmental samples, they may not be best suited for all food varieties (Heller et al. 2003; Schuurman et al. 2005; Wolffs et al. 2004). The inhibition of amplification may be because of a number of factors, none of which has been investigated thoroughly (Grant 2003; Wilson 1997). To date, only a small number of studies have successfully used alternative methods to remove the PCR inhibitor for real-time PCR detection of food-borne pathogens such as filtration or buoyant density gradient separation (Wolffs et al. 2006; Fukushima et al. 2007; Rodriguez-Lazaro et al. 2004b, 2005). To increase the specificity of detection, a number of PCR assays utilize post-PCR hybridization methods (Weagant et al. 1999; Rijpens et al. 1999; Cocolin et al. 1998; Chen and Griffiths 2001). These approaches have met with limited success, as these modifications make the overall pathogen detection procedure labor-intensive, time-consuming, and difficult to automate.

Practicality of using a filter-based DNA extraction method (FTA) (Burgoyne 1996) for template preparation and subsequent use in real-time PCR assays was examined in this study. The protocol uses filter paper impregnated with a patented chemical formulation that lyses cells, immobilizes DNA, and protects it from degradation. The technique was originally designed for DNA isolation and storage from blood (Burgoyne 1996) and has been used to isolate DNA from parasitic protozoa and human pathogens for use in conventional PCR assays (Nantavisai et al. 2007; Lampel et al. 2000). We reasoned that FTA filters would offer more rapid and sensitive protocol to simultaneous separation of plant phenolics and other PCR inhibitory compounds from food matrixes and lysis of food-borne pathogens. With FTA filters, released DNA is sequestered and preserved intact within the membrane (Burgoyne 1996; Nantavisai et al. 2007; Lampel et al. 2000). After a series of washes, filters can be used directly in conventional PCR assays with comparable detection sensitivity (Orlandi and Lampel 2000). Successful implementation of the FTA protocol for the detection of low level of human pathogens on fresh produce would carry distinct advantages of rapidity, simplicity, and biosafety. Moreover, the procedure can be carried out under field conditions with minimal laboratory equipments. Whereas several investigators have used FTA and other filter types for DNA isolation (Orlandi and Lampel 2000; Oyofo and Rollins 1993), its suitability for use in real-time PCR has not been investigated. This study examined three commercially available DNA isolation procedures, namely, BAX DNA lysis, Bio-Rad iQ-Check, and FTA filter cards, which are commonly used for PCR-based pathogen detection systems (Bhagwat et al. 2008; Orlandi and Lampel 2000; Shearer et al. 2001; Oravcova et al. 2007), were compared. The protocols were evaluated for the detection of *Salmonella* spp. and *Listeria monocytogenes* in fresh and fresh-cut produce artificially inoculated at a level of <10 (approximately four to seven) colony forming units (CFU) per 25 g.

Materials and Methods

Bacterial strains and media *L. monocytogenes* ATCC 13932 and *Salmonella enterica* serovar Typhimurium SL1344 were used as reference strains (Gawande and Bhagwat 2002; Shen et al. 2006; Fang et al. 1992). Cultures were routinely initiated from freezer stocks for growth on tryptic soy agar (TSA) medium (Difco Laboratories, MI, USA). After overnight incubation at 37 °C, a single colony was selected and inoculated into 10 ml tryptone soya broth in a 125-ml Erlenmeyer flask. Cultures were grown for 20 to 22 h at 37 °C with shaking at 200 rpm (Lab-Line Instruments, IL, USA) to obtain stationary-phase cultures. The cells were harvested by centrifugation at 10,000×*g* for 10 min (Eppendorf 5410R, Hamburg, Germany), washed once with three volumes of saline (0.85% NaCl), and suspended in saline at a cell density of 10^9 cells per ml. Cells were further diluted in saline to achieve the desired cell density. Final cell numbers were confirmed by determining viable cell counts on TSA plates.

Inoculation of fresh produce Fresh produce (alfalfa sprouts, cilantro, green onion, broccoli, prepacked mixed salad [made up of approximately 80% leaf lettuce, 10% red cabbage, and 10% carrot by weight], and spinach) was obtained from local grocery stores and examined for the

presence of *Salmonella* and *L. monocytogenes* using conventional U.S. FDA-BAM microbiology protocol 34.

Fresh produce free of pathogens was artificially inoculated with either *S. enterica* serovar Typhimurium or *L. monocytogenes* at a low inoculum dose of four to seven CFU per 25 g for this investigation. Each experiment was performed at least three times and each experiment contained three subsamples per produce. Inoculation was performed with approximately four to seven cells in 50 μl saline using pure cultures of either *S. enterica* serovar Typhimurium or *L. monocytogenes* and processed within 10 min for enrichment. For each experiment, noninoculated produce was processed along with rest of the samples and was found to be free of *S. enterica* serovar Typhimurium and *L. monocytogenes* by PCR and culture methods.

Enrichment procedures Experimentally inoculated samples were subjected to enrichment protocols as described previously specific for *Salmonella* spp. (Liming and Bhagwat 2004) and *L. monocytogenes* (Liming et al. 2004). Briefly, for samples inoculated with *L. monocytogenes*, 25 g of produce was combined with 225 ml of one half strength Fraser broth in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender. The samples were incubated for 24±2 h without shaking at 30 °C. After the incubation period, 1 ml of the sample was withdrawn from the top without disturbing the food debris and processed for DNA isolation (Liming et al. 2004). For samples inoculated with *S. enterica* serovar Typhimurium, 25 g of produce was combined with 225 ml of buffered peptone water (Difco Laboratories, Detroit, MI, USA) in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender. The samples were incubated for 24±2 h without shaking at 37 °C. After the incubation period, 1 ml of the sample was withdrawn and processed for DNA isolation (Liming and Bhagwat 2004).

DNA extraction procedures For each pathogen, bacterial DNA was extracted using three different procedures. For the BAX lysis method, 1 ml samples from the enrichment broth were centrifuged at 10,000×*g* for 5 min, and the pellet was suspended in 200 μl of BAX cell lysis reagent (Qualicon, Wilmington, DE, USA). DNA was then isolated according to the manufacturer's protocol. Briefly, cells were incubated in the BAX lysis buffer for 1 h at 55 °C and then for 10 min at 95 °C (to inactivate the proteases in the lysis buffer) (Bhagwat 2003). For the iQ-Check protocol, 1 ml samples from the enrichment broth were centrifuged at 10,000×*g* for 5 min, and the pellet was suspended in 200 μl of lysis reagent and vortexed. Lysis was carried out by incubating the suspension at 100 °C for 15 min (Bhagwat et al. 2008). For FTA filter-based DNA isolation, 1 ml samples from the enrichment broth were centrifuged for 10,000×*g* for 5 min, and the pellet was suspended in 65 μl of Tris buffer (10 mM Tris–HCl, pH 8.0), applied to the FTA card, and processed as per the manufacturer's instruction (Whatman, Trenton, NJ, USA). Briefly, the filter was dried at room temperature for at least 1 h and washed once with FTA purification reagent provided by the manufacturer followed by a rinse in TE buffer. Air dried filters were kept in an air-tight container for long-term (3–6 months) storage to test the sample stability.

For isolating DNA from pure cultures of *L. monocytogenes* and *S. enterica* serovar Typhimurium, a tenfold serial dilution of known quantities of viable cells (10^9–10^5, grown in tryptone soya broth and measured as CFU ml^{-1}) were mixed in individual tubes containing lysis buffer and used as standards in the respective PCR assays.

Detection of L. monocytogenes and S. enterica serovar Typhimurium by real-time PCR For the iQ-Check PCR protocol, samples were examined in duplicate at two concentrations of template DNA. For each sample, 5 μl of 1:10 and 1:25 diluted DNA were mixed with 40 μl of amplification mixture and 5 μl of fluorogenic oligonucleotide molecular beacon probe solution. For FTA protocol when cells in 65 μl volume were spotted on filter disc, 2.0 mm discs area yielded DNA equivalent to the quantity in 5 μl lysis buffer from either BAX or iQ-Check protocols. Three 2.0 mm discs were punched from each FTA card using a Uni-Core punch provided by the manufacturer and used in three PCR reactions.

For *L. monocytogenes* detection, the probe was labeled with Texas Red at the 5′-end and DABSYL at the 3′-end as the quencher. The fluorogenic MB-probe from the iQ-Check kit (Bio-Rad Laboratories) targets an *L. monocytogenes*-specific region of the *hly* gene. To monitor successful DNA amplification in each reaction tube, the kit provides a synthetic DNA (at a low concentration) as a part of the reaction mixture which works as an internal control. This control DNA was amplified with a specific probe at the same time as the *L. monocytogenes* target DNA sequence and detected by a second fluorophore (FAM). The thermocycler (iCycler, Bio-Rad Laboratories) was programmed for 50 °C for 2 min, 95 °C for 5 min (95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s) for 50 cycles, and 72 °C for 5 min.

For *S. enterica* serovar Typhimurium detection, a fluorogenic oligonucleotide molecular beacon probe was labeled with FAM at the 5′-end and DABSYL at the 3′-end as the quencher. The fluorogenic MB-probe from iQ-Check kit (Bio-Rad Laboratories) targets the *i*nvasion *a*ssociated gene (*iagA*) which is highly specific to *Salmonella* species (Miras et al. 1995). The internal control DNA was amplified with a specific probe at the same time as the *Salmonella* target DNA sequence and detected by a second

fluorophore (Texas Red). The thermocycler was programmed for 50 °C for 2 min, 95 °C for 5 min (95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s) for 50 cycles, and 72 °C for 5 min.

Detection of L. monocytogenes and S. enterica serovar Typhimurium by conventional microbiology methods The protocol recommended for *L. monocytogenes* by the U.S. FDA-BAM was followed (Shen et al. 2006). After an enrichment step, the broth was streaked on Bacto-modified Oxford agar (MOX) and Bacto-PALCAM agar (Difco laboratories) to identify *L. monocytogenes*. Similarly, *Salmonella* strains were detected with the U.S. FDA-BAM protocol (Bhagwat et al. 2008). After enrichment, the identity of *Salmonella* strains was confirmed by the occurrence of black colonies on *Salmonella–Shigella* (SS) agar and black colonies with a bright metallic sheen on Bismuth sulfite (BS) agar (Bopp et al. 1999).

Results and Discussion

The data from Fig. 1 show the threshold cycle (C_t) values for real-time PCR performed using DNA isolated by iQ-Check, BAX, and FTA methods. The BAX DNA isolation protocol (Shearer et al. 2001; Bhagwat 2003) was included as this method is being used by the microbiological data program (MDP) of USDA's Agricultural Marketing Service which targets sampling of fresh produce (Jofre et al. 2005) in participating states for pathogenic *E. coli*, *Salmonella*, and *E. coli* O157:H7 (Bhagwat 2006). A no-template-control in which sterile saline was substituted for template DNA was used in each experiment. This control was used to subtract any fluorescence that was not directly related to amplification. For the *S. enterica* serovar Typhimurium template DNA prepared by all three procedures, C_t values decreased linearly with increasing the target quantity from 10^1 to 10^6 cells per PCR assay (Fig. 1a) with correlation coefficients of 0.996 (iQ-Check templates), 0.985 (BAX), and 0.962 (FTA filters). The amplification plot generated a slope of −3.83 and −3.5 (for iQ-Check/BAX and FTA templates, respectively), corresponding to ~83% efficiency of the PCR assay, using the formula, efficiency (E)=($10^{-1/\text{slope}}$)−1 (Higuchi et al. 1993). These values are very similar to the previously reported data where a mixture of five *Salmonella* serovars (i.e., Agona, Anatum, Dublin, Haifa, and Choleraesuis) yielded a correlation coefficient value of 0.98 when DNA template was prepared using iQ-Check lysis buffer (Liming and Bhagwat 2004). Similarly, detection of *L. monocytogenes* DNA prepared by the three methods generated an inverse linear relationship between C_t values and starting template concentration (Fig. 1b). DNA prepared

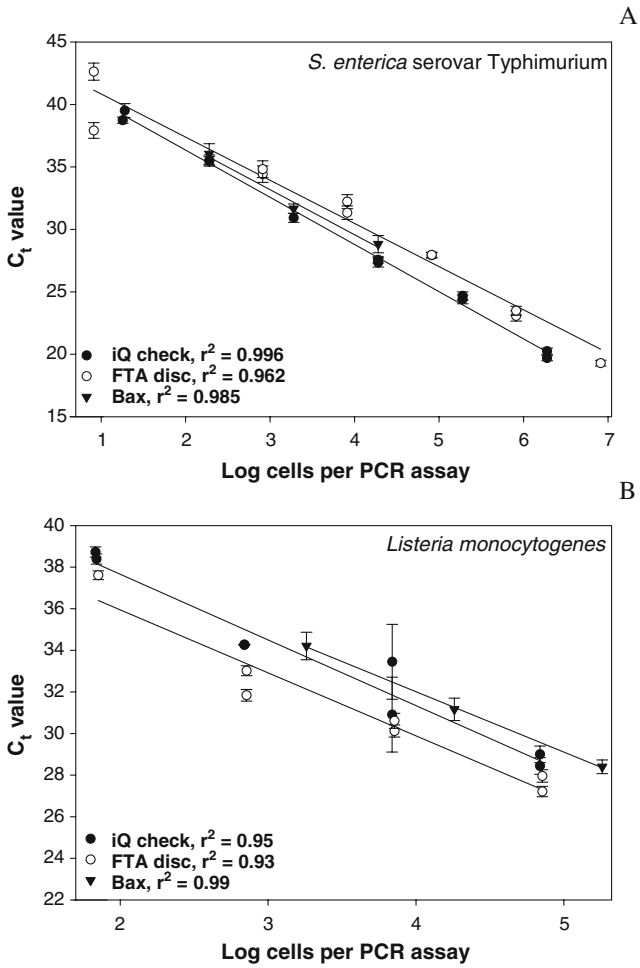

Fig. 1 Standard curve for a tenfold serial dilution series of *S. enterica* serovar Typhimurium (**a**) and *L. monocytogenes* (**b**) plotted as the threshold cycle (C_t) on the *Y*-axis using template DNA prepared by three different methods. The target copy number per assay is on the *X*-axis, DNA was prepared by iQ-Check (*filled circles*), BAX (*filled inverted triangles*), and FTA filter discs (*open circles*) protocols

by all three template preparation procedures showed a similar slope with high correlation coefficients of 0.95, 0.99, and 0.93 (iQ-Check, BAX, and FTA templates, respectively). Thus, the presence of FTA filter disc in the PCR assay tube did not interfere with the real-time detection of the two reporter dyes for *Salmonella* and *L. monocytogenes*. The log-linear relationship of C_t values and viable cells obtained using DNA template from FTA filters was in agreement with previously reported data (Liming and Bhagwat 2004; Liming et al. 2004).

Furthermore, the effectiveness of three template DNA preparation methods on the detection of *Salmonella* sp. and *L. monocytogenes* in real-time PCR assays from fresh-cut produce was tested using artificially contaminated alfalfa sprouts, cilantro, green onion, broccoli, prepacked mixed salad, and spinach. The PCR assays and conventional microbiological protocols were performed in parallel for

Table 1 Comparative analyses of detection frequencies of *S. enterica* serovar Typhimurium and *L. monocytogenes* from artificially inoculated fresh produce by real-time PCR using three template preparation methods

Fresh produce	*Salmonella enterica* serovar Typhimurium				*Listeria monocytogenes*			
	FDA-BAM culture method	Real-time PCR template prepared by			FDA-FSIS culture method	Real-time PCR template prepared by		
		Bax lysis	iQ-Check	FTA filter		Bax lysis	iQ-Check	FTA filter
Alfalfa sprouts	11/11	5/11	11/11	11/11	6/6	2/6	3/6	6/6
Cilantro	9/9	8/9	9/9	9/9	9/9	4/9	7/9	9/9
Green onion	7/7	3/7	7/7	7/7	7/7	5/7	7/7	7/7
Broccoli	12/12	12/12	12/12	12/12	7/7	7/7	7/7	7/7
Mixed salad	15/15	15/15	15/15	15/15	8/8	8/8	8/8	8/8
Spinach	8/8	7/8	8/8	8/8	6/6	6/6	6/6	6/6

each produce sample. For each experiment, noninoculated produce was used as a control and was found to be free of *Salmonella* spp. and *L. monocytogenes* by PCR and culture methods. Using the microbiological methods (see the "Material and Methods" section), we were able to detect contamination of both pathogens from all produce at a low level of contamination in all samples (<10 CFU—approximately four CFU per 25 g produce; Table 1).

On the other hand, detection of pathogens using real-time PCR was found to be dependent upon template DNA preparation protocol (Table 1). Although samples were withdrawn from the same enrichment media, the ability of DNA to serve as a template in real-time PCR varied with the produce and isolation protocols. Plant pigments, phenolics, and polysaccharides are known to interfere with PCR and their inhibitory influence on DNA polymerases has been documented (Schuurman et al. 2005; Shearer et al. 2001; Liao and Shollenberger 2003). In this study, we observed that broccoli, mixed salad, and spinach gave the most consistent results with all of the three template preparation methods and low levels of *Salmonella* and *L. monocytogenes* contamination (<10 CFU—approximately four to seven CFU per 25 g produce) were successfully detected. However, for the detection of *L. monocytogenes* on alfalfa sprouts and cilantro, when template DNA was isolated with either BAX or iQ-Check method, several samples gave false-negative results although the samples tested positive when analyzed using selective agar growth media (Table 1). When FTA-based protocol was followed, *L. monocytogenes* was detected with improved frequency. Similarly, *Salmonella* detection frequencies with artificially contaminated green onion using BAX lysis buffer were less than 50% as four out of seven samples gave false-negative results. In assays where false-negative data were obtained, there was also no amplification of the internal template DNA even after 1:25 dilution of the template DNA (data not shown), indicating inhibition of the reaction. When analyzed by the conventional selective media all samples tested positive. It is interesting to note that the iQ-Check DNA template preparation protocol and FTA protocol yielded positive results for all green onion samples. Using the InstaGene matrix, Fortin et al. (2001) reported improved detection frequency for *E. coli* O157:H7 in raw milk samples. Likewise, using a similar reagent (i.e., Chelex-100-based DNA purification method) a detection limit of 100 CFU/g of meat was observed for *L. monocytogenes* (Rodriguez-Lazaro et al. 2004b). Comparison of four different commercial DNA preparation protocols (Prepman Ultra, NucleoSpin, Bugs'nBeads, and Wizard DNA purification systems) by real-time PCR of artificially contaminated foods yielded similar detection limit for all four methods (Heller et al. 2003). In that study the detection limit was estimated to be 5.3×10^3 *E. coli* O157:H7 cells/g of salad green or ground beef which translates to 1.3×10^5 cells/25g of food (Heller et al. 2003). The detection limits observed using FTA filters are similar to those observed by Fortin et al. (2001) where one CFU of *E. coli* O157:H7 per milliliter of raw milk or apple juice was detected using molecular beacon probe real-time PCR after enrichment.

We also observed that both pathogens were inactivated within 10 min once in contact with FTA filters and a >5-log kill was observed when viability was tested 1 h after application of bacteria from the enrichment broth (data not shown). After 5 h, no viable bacteria could be recovered from the FTA filters making it safe for transportation without any of the restrictions normally associated with live pathogens or cold storage for DNA isolated by BAX or iQ-Check procedures.

In conclusion, as demonstrated in this study, we expanded the utility of FTA filters to include the detection of *Salmonella* and *L. monocytogenes* by real-time PCR. The DNA isolation protocol was successful in generating PCR templates pure enough to detect the pathogens from the artificially contaminated leafy green produce otherwise

known to possess strong PCR inhibitory compounds. The use of FTA filters eliminated the need for additional equipment that may be necessary to isolate DNA by other methods such as silica gel filtration or heating blocks and saved time needed to perform multistep procedures. FTA filters offer an alternative to laborious and often cumbersome isolation and purification schemes which are tedious to carry out under field conditions.

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